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Production of Surface-Active Lipids by *Corynebacterium lepus*

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Corynebacterium lepus was grown in 20-liter batch fermentations with kerosene as the sole carbon source. Critical micelle concentration measurements indicated the production of appreciable quantities of biosurfactants. This surface activity of the culture medium was due to lipids, which were extracted and identified. Samples of *C. lepus* whole broth were taken during a fermentation and monitored for surface tension, amount of surfactant present, and lipid content. The changes in the surfactant measured correlated with concentration changes of several surface-active lipids. An early dramatic increase in surfactant concentration was attributed to the production of a mixture of corynomycolic acids (β-hydroxy α-branched fatty acids). Surface activity at the end of the fermentation was due to a lipopeptide containing corynomycolic acids plus small amounts of several phospholipids and neutral lipids which were identified by thin-layer chromatography.

Several species of microorganisms are reported to produce surface-active agents (1, 3–6, 9–12, 17, 20, 21). These are usually organisms which produce surface-active lipids when growing on hydrocarbon substrates. The surface activity is observed as a substantial lowering of the culture medium surface tension, an emulsification of the medium and hydrocarbons, or related phenomena. Several different types of biosurfactants have been isolated and characterized. These include glycolipids (6, 9, 11, 21), lipopeptides (1, 10), phospholipids (3, 12), and neutral lipids (17, 20). The complex lipids all contain fatty acids (1, 3, 6, 9–12, 21), and often these fatty acids have a hydroxyl function on the carbon β to the carboxyl group (1, 10) or farther along the chain (6, 9, 11).

Gerson and Zajic (5) reported the isolation of *Corynebacterium lepus* which will grow on kerosene. During growth, the surface tension of the medium quickly fell from 72 dyn/cm to under 30 dyn/cm, and the active agent was produced in quantities greatly in excess of the critical micelle concentration (CMC; concentration of surfactant which causes micelle formation). With the best samples it was necessary to dilute portions of *C. lepus* culture medium 200 to 500 times before the surface tension rose above 30 dyn/cm. Gerson and Zajic (5) also reported that the CMC and, by implication the concentration of biosurfactant present, varied dramatically during batch fermentations of *C. lepus* on kerosene.

This paper reports the nature of the surface-active lipids produced by *C. lepus* and their changes in concentration throughout a fermentation.

MATERIALS AND METHODS

Fermentation studies. Gerson and Zajic (5) described the isolation, identification, and fermentation of *C. lepus*. For this work a 28-liter New Brunswick fermentor was used as described previously (5). The temperature was controlled at 25°C. Air was introduced with a 50-mm stainless steel disk at 20 liters/min and a pressure of 4 lb/in^2. A 25-ml inoculum in the early stationary phase was used. The media used for both the fermentations and the inocula were made with standard mineral salts media (5). They contained the following ingredients: $NaNO_3$, 0.2%; K_2HPO_4, 0.1%; KH_2PO_4, 0.05%; KCl, 0.01%; $MgSO_4$, 0.05%; $CaCl_2$, 0.001%; $FeSO_4$, 0.001%; ethylenediaminetetraacetate, 0.00015%; and trace amounts of B, Cu, Mn, Mo, and Zn. The medium usually contained 4% Imperial Oil no. 9 kerosene and 0.01% nutrient broth.

Extraction of lipids from lyophilized *C. lepus* product. Samples of culture medium (ca. 60 g) of *C. lepus* were centrifuged to give an emulsion at the interface between the aqueous medium and kerosene. This emulsion was collected by suction and lyophilized. Samples taken at the end of a fermentation yielded 2 to 3 g of a friable off-white solid per liter.

Lipids were extracted from this solid by using a standard method (reference 13, p. 351). The product was stirred in chloroform–methanol–water (25:25:4; 10 ml per g of product) for 6 h. An equal volume of water was added, and the mixture was stirred vigorously for 1 h. The organic phase was isolated, washed with

water, and evaporated to dryness. The resulting yellow oil was extracted with acetone at room temperature.

The acetone-soluble fraction was obtained as a yellow oil after evaporation of the solvent. A typical yield was 2 to 5% of the original dry weight of product collected from the culture medium.

The acetone-insoluble fraction was a pale waxy solid which contained polar lipids. This was dissolved in chloroform and filtered with Whatman 541 filter paper, and the solvent was removed. This was the major lipid fraction, and typically the yield was 20 to 25% of the original dry weight.

Extraction of lipids from samples of culture medium. This method was used for monitoring lipids throughout a fermentation. Samples of medium (ca. 60 g) were weighed, shaken with 20 ml of pentane, and centrifuged. The pentane above the product emulsion was removed, and the extraction was repeated. The pentane was evaporated from the combined extracts, and the residue was lyophilized. The resulting oil was transferred to a 25-ml volumetric flask and made up to volume with pentane.

The floating solid remaining in the pentane-washed samples was removed and lyophilized. After the dried product was weighed, it was extracted with chloroform–methanol–water as described above.

Extraction of lipopeptides from culture medium. It was possible to obtain a lipopeptide fraction by first adding sufficient HCl to a culture medium sample to lower the pH to 2 and then extracting with chloroform. After the chloroform was removed, the residue was washed with acetone and then lyophilized. The lipopeptide was obtained as a white powder in yields of up to 0.35 g/liter.

Calculation of CMC. The CMC is defined as the concentration of a surfactant necessary to initiate micelle formation. If more of the surfactant is present, there will be no further decrease in surface tension (5). To obtain a measure of the CMC of a whole broth sample, portions were diluted by various amounts, and the surface tension was obtained for each dilution. The CMC could then be estimated, from a plot of surface tension versus the log of the percent dilution, as the dilution at which the surface tension starts to increase. The reciprocal of the CMC (CMC^{-1}) is proportional to the amount of surfactant present.

TLC. Thin-layer chromatography (TLC) plates, both analytical and preparative, were prepared with Merck G silica gel. Plates (20 by 20 cm) were spotted with lipid extracts and known compounds and developed in one of the following: solvent 1, chloroform–methanol–water (65:25:4); solvent 2, chloroform–methanol–acetic acid–water (25:15:4:2); solvent 3, chloroform–acetone–methanol–acetic acid–water (7:8:2:2:1); or solvent 4, hexane–isopropyl ether–acetic acid (15:10:1). After developing, the spots were visualized with standard spray reagents (reference 13, p. 435–441). Ninhydrin generated a red or purple color when a compound had an amine function. The Zinzadze reagent reacted with phosphate-containing lipids, resulting in a blue color. Spraying the plate with an α-napthol solution, followed by concentrated sulfuric acid and then heating for 10 min, indicated carbohydrate by a red color. Certain compounds appeared as fluorescent spots under UV irradiation. Neutral lipids which were not indicated by the above methods were visualized by spraying with rhodamine 6 G and observed under UV irradiation as yellow spots. All of the lipids could be visualized by charring the plates after spraying with potassium dichromate in sulfuric acid. Identifications were made on the basis of comparisons with published data (4, 7, 13, 15) and corroborated with available phospholipids, including phosphatidyl inositol, phosphatidyl serine, phosphatidyl glycerol, phosphatidic acid, phosphatidyl choline, and lysophosphatidyl choline.

Instrumentation and chemicals. Infrared spectra were obtained on a Beckman IR-20 spectrometer by using chloroform solutions. UV and visible spectra were obtained on a Perkin-Elmer spectrometer by using pentane solutions. Protein was determined by using the biuret method (reference 8, p. 244–249). Amino acids were determined with a Beckman amino acid autoanalyzer. Solvents used were Fisher Scientific Co. Spectranalyzed grade. Standard lipids were obtained from Serdary Research Laboratories Inc., London, Ontario, Canada.

RESULTS

Variations in surface tension and CMC^{-1} during a fermentation of *C. lepus*. Figure 1 contains some of the data from a typical fermentation of *C. lepus*, plotted as a function of sampling time. The surface tension of the culture medium quickly dropped to below 30 dyn/cm and remained constant to the end of the fermentation. The plot of the reciprocal of the CMC, which is a measure of surfactant concentration, shows more variation. Initially there was insufficient surfactant present to form micelles. At 25 h the CMC^{-1} started to rise sharply, reaching a maximum at about 44 h. It then decreased by more than an order of magnitude and remained constant after 60 h. Although not observed in this example, during many of the fermentations the CMC^{-1} had a second, small increase after about 70 h. The biosurfactant never completely disappeared in any of the fermentations monitored.

Biosurfactants present at the end of the fermentation. Initially, a study was made of the compounds present in the culture medium at the end of the fermentation. This corresponds to the region in Fig. 1 after 60 h when the plot of CMC^{-1} had leveled off. The surface activity of the culture medium was found to be due to the fractions soluble in lipid solvents. These were concentrated in a slimy white solid produced in copious quantities by the end of the fermentation (ca. 3 g/liter). After removal of the product emulsion, the residual medium had a surface tension greater than 60 dyn/cm, and the amount of remaining surfactant was less than the CMC. If the dried product was redissolved in distilled water, the minimum surface tension

was 49 dyn/cm. This was not as low as that obtained for the whole broth; however, the whole broth contained sufficient kerosene to influence the surface tension measurements. Samples of sterile medium did not yield detectable amounts of lipids.

The surface-active lipids were extracted from the dried product (0.5 g/liter of original whole broth; minimum surface tension, 49 dyn/cm) and partitioned into acetone-insoluble polar lipids (88%) and acetone-soluble neutral lipids (12%).

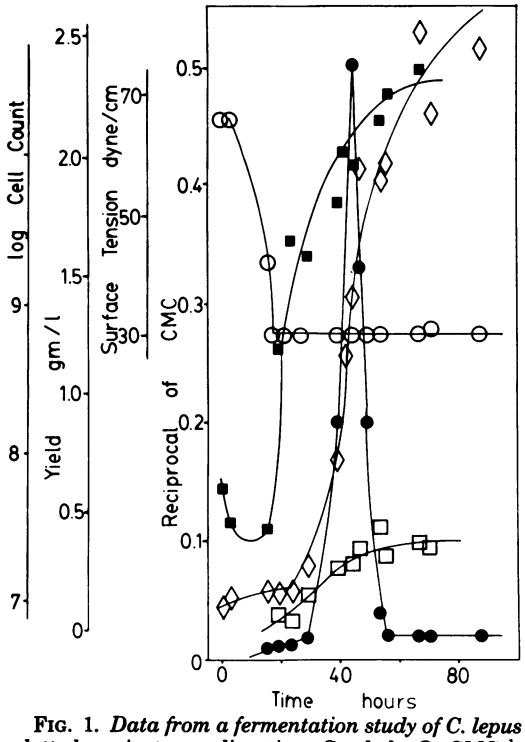

FIG. 1. *Data from a fermentation study of C. lepus plotted against sampling time. Symbols: ●, CMC^{-1}; ○, surface tension; ◇, yield of floating product; □, yield of polar lipids; and ■, log of the cell count.*

The polar lipids were resolved into several compounds by using TLC (Table 1). Six phospholipids were observed. Phosphatidyl glycerol, phosphatidyl inositol, and a carbohydrate-positive phospholipid, which is probably one of the phosphatidyl inositol mannosides often found in coryneform bacteria (7, 15, 16), were present in roughly equal amounts, and all were easily distinguished. Phosphatidyl glycerol phosphate and cardiolipin could not be resolved at the end of the fermentation, but the study of lipids present throughout a fermentation indicated that both were present (vide infra). Only a very small amount of phosphatidyl serine was observed. A single unidentified glycolipid was observed.

The above lipids accounted for only a small amount of the polar lipid fraction. At least 90% of the acetone-insoluble residue was a mixture of ninhydrin-positive lipids. These lipopeptides were a relatively long smear on the TLC plates, and there were probably more than one present. The lipopeptides could be obtained free of the other polar lipids by acidifying a sample of culture medium and extracting it with chloroform. The minimum surface tension for the lipopeptide added to distilled water was 52 dyn/cm.

A biuret determination indicated a protein concentration of about 35% by weight. Table 2 shows the amino acid composition. About 20% of the residues present were acidic (i.e., glutamic or aspartic acids), and most of the rest were neutral amino acids.

The remainder of the lipoprotein was found to be 25% saturated fatty acids and 75% corynomycolic acids. The isolation and characterization of these acids will be reported in full in a separate

TABLE 1. *TLC data for lipids isolated from C. lepus*

Lipid	R_f in solvent:			Phosphate test	Amine test	Carbohydrate test	Time (h) of appearance[a]
	1	2	3				
Lipopeptides	0	0.04–0.15	0.07–0.22		+		21
Phosphatidyl glycerol	0.52	0.8	0.75	+			19
Phosphatidyl inositol	0.22	0.5	0.3	+			19
Phosphatidyl inositol mannoside	0.55	0.58	0.22	+		+	19
Phosphatidyl serine	0.17	0.63	0.51	+	+		20
Cardiolipin[b]	0.7	0.94	0.90	+			27
Phosphatidyl glycerol phosphate[b]		0.90		+			20
Glycolipid	0.57	0.35	0.5			+	27
Neutral lipids	0.5–0.75	1.0	1.0				30–45

[a] From a monitored fermentation; inoculation was zero time.

[b] These two phospholipids were not resolved except in samples taken at from 20 to 50 h.

publication. Corynomycolic acids are β-hydroxy α-branched fatty acids which have been isolated previously from coryneform bacteria (2, 16). The mixture of fatty acids obtained by saponification (alcoholic potassium hydroxide; reference 13, p. 363) had a very low minimum surface tension in distilled water (32 dyn/cm).

The acetone-soluble neutral lipids were only about one-tenth of the total lipid extract but had a relatively low surface tension in water (40 dyn/cm). Table 3 lists the components which were identified in this fraction. The various glycerides were present in very small amounts and were only identified by comparison on the TLC plates with known compounds. The other components were present in sufficient amounts to obtain samples by preparative TLC and to confirm identification by infrared spectroscopy of chloroform solutions. The most prominent spot on the plates was usually due to corynomycolic acids (νOH, alcohol 3,530 cm^{-1}; νOH, carboxyl 3,000 and 2,700 cm^{-1}; νC=O 1,710 cm^{-1}; νC—O 1,100, 1,290 cm^{-1}). There were also smaller amounts of simple fatty acids and a compound with νC=O at 1,740 cm^{-1}, which was tentatively identified as a ketone (e.g., neither νC—O, expected for an ester, nor νC(O)—H, expected for an aldehyde, were observed [13]). Finally, there was a significant component moving with the solvent front, which was identified as a nonvolatile kerosene residue.

TABLE 2. *Amino acids found in lipopeptide from C. lepus*

Amino acid	Mole composition[a] (%)
Alanine	17.4
Glutamic acid	15.6
Glycine	13.0
Leucine	10.6
Serine	8.4
Threonine	7.7
Phenylalanine	6.4
Aspartic acid	5.9
Proline	5.5
Methionine	3.0
Isoleucine	2.6
Valine	2.1
Lysine	1.8

[a] Expressed as a percentage of the total moles of amino acids in the sample.

TABLE 3. *TLC data for neutral lipids isolated from C. lepus with solvent 4*

Lipid	R_f	Identification[a]	
		Standard	Infrared
Monoglyceride	0.03	+	
1,2-Diglyceride	0.2	+	
1,3-Diglyceride	0.25	+	
Corynomycolic acids	0.3–0.4		+
Fatty acids	0.6	+	+
Triglyceride	0.7	+	
Ketone	0.85		+
Kerosene residue	0.9–1.0	+	+

[a] Lipids were identified by comparison with standard lipids and by infrared spectroscopy when possible. The four glyceride references were obtained from olive oil, and palmitic acid was used as a fatty acid.

Variation in surfactant concentration during a fermentation of *C. lepus*. In an attempt to explain the behavior of the CMC^{-1} data during a fermentation, samples of the culture medium were taken at various times during the fermentation shown in Fig. 1. These were analyzed for changes in surface-active lipids. The extraction procedure for samples of whole broth outlined above resulted in three types of samples: (i) the aqueous supernatant, (ii) the pentane extract, and (iii) the lipid extract of the floating product.

(i) Aqueous supernatant. The aqueous phase which had been exhaustively extracted for lipids showed little surface activity throughout the fermentation. The surface tension did not go below 60 dyn/cm. The optical density at 270 nm of the aqueous phase increased throughout the fermentation due to the presence of a small but steadily increasing amount of protein. As this did not contribute significantly to the surface tension of the whole broth, it was not studied further.

(ii) Pentane extracts. Pentane solutions of the neutral lipids were used for the following measurements. As it was impossible to take exactly the same weight of whole broth with each sample, when feasible the data were normalized relative to the measured sample weights. If this was not possible due to the nature of the measurement, only data from samples which weighed 60 ± 5 g were considered. The surface tension data were obtained from a distilled water solution prepared by layering 1 ml of the pentane solution on 5 ml of water and evaporating the pentane in a stream of air.

A plot of CMC^{-1} from the pentane-soluble lipids versus sampling time (Fig. 2) is similar to the plot of CMC^{-1} of culture medium (Fig. 1). At about 25 h it began to increase sharply, reaching a maximum between 40 and 45 h. It then decreased by an order of magnitude by 60 h. The pentane samples at this peak in surfactant concentration had a noticeable yellow color which was not seen earlier or later in the fermentation. The optical densities of the pentane solutions at 258 nm are also plotted in Fig. 2.

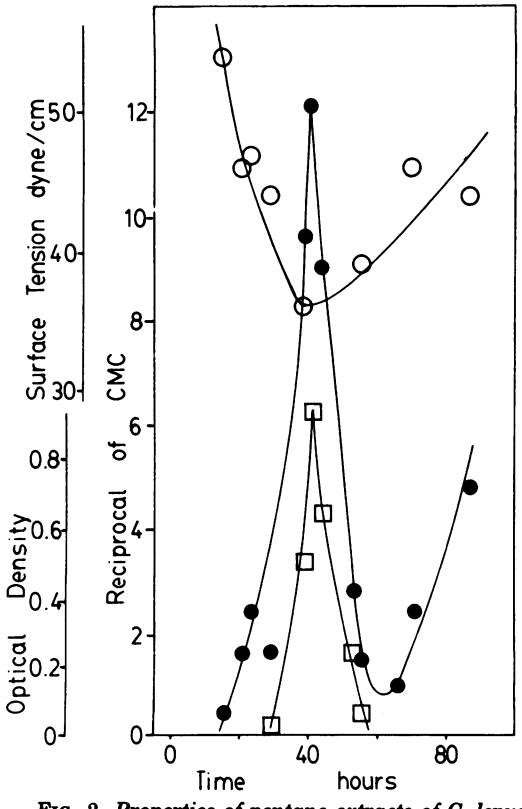

FIG. 2. *Properties of pentane extracts of C. lepus fermentation samples versus sampling time. Symbols: ●, CMC^{-1}; ○, surface tension; and □, optical density.*

The peak in optical density closely mimics the peak in CMC^{-1}. However, the absorbing species disappeared completely by 60 h. The CMC^{-1} plot indicated that some surfactant had reappeared, as it displayed a significant increase after 60 h. The final plot in Fig. 2 is the initial surface tension before diluting the solutions to obtain CMC data. This curve is not as dramatic as the first two. However, there was a minimum in surface tension (36 dyn/cm) at the same time as the maxima in CMC^{-1} and optical density. The surface tension then increased to about 46 dyn/cm at the end of the fermentation.

Micropipettes were used to spot equal amounts of several pentane solutions along one edge of a single TLC plate which was then developed in solvent 4. Relatively small samples were applied to the plates, and, even at the end of the fermentation, most of the neutral lipids recorded in Table 3 were not observed. Corynomycolic acids were observed after spraying the plates with rhodamine 6 G. They first appeared in the sample taken at 29 h and reached a peak in concentration between 40 and 45 h. They then decreased in concentration, although they were still observed as a faint spot by the end of the fermentation. Two other compounds were observed as fluorescent blue spots under UV irradiation before spraying with rhodamine 6 G (R_f's in solvent 4, 0.75 and 0.85, respectively). Both first appeared before 30 h, reached a maximum concentration between 40 and 45 h, and were not detected by 60 h. As neither was present at the end of the fermentation, they had not been observed in the preliminary study of the neutral lipids (Table 3). Even with preparative TLC of the samples taken at 40 to 45 h, it was impossible to obtain sufficient quantities of these two compounds to identify them.

The final analysis of the pentane samples was to concentrate portions and use these to spot TLC plates in an attempt to observe the other neutral lipids. Although this made it more difficult to observe the behavior of individual lipids, all of the components listed in Table 3 were present by 40 h. For each lipid there was a general increase in concentration to a plateau after 60 h. This was synchronous with the second increase in CMC^{-1} and the leveling-off of the surface tension shown in Fig. 2.

(iii) Lipid extract of floating product. The yield of floating product increased steadily throughout the fermentation of *C. lepus* (Fig. 1). The yield of polar lipid extracted from the floating product also increased throughout the fermentation (Fig. 1). There was no evidence for maxima in these concentration curves corresponding to the maximum in the CMC^{-1} plot of the whole broth. Instead, the maximum concentrations were observed at the end of the fermentation.

The polar lipid samples were subjected to a comparative TLC study similar to that described for the pentane extracts except that solvent 2 was used. No lipids were observed which had not been identified previously in the products collected from *C. lepus* fermentations in late growth phase (Table 1). Table 1 also lists the time of first appearance of each polar lipid. The first were observed at 19 h and by 30 h all of the phospholipids, the lipopeptides, and the glycolipid had appeared. There were no apparent concentration maxima for the polar lipids between 40 and 45 h, as was observed for some of the neutral lipids. Instead, the polar lipid concentrations increased steadily until about 70 h, after which they appeared to remain constant.

Monitoring the phospholipid concentrations as a function of fermentation time confirmed the occurrence of both phosphatidyl glycerol phosphate and cardiolipin, which could not be resolved in the initial TLC studies. Phosphatidyl

glycerol phosphate was observed first at 20 h as a small phosphate-positive spot at R_f 0.90. At 27 h a second small spot appeared at R_f 0.94 touching the original slightly lower spot, and this was attributed to cardiolipin. As these two spots increased in size in later samples, they coalesced into a single spot.

DISCUSSION

All of the surface-active agents isolated from *C. lepus* were lipids. The CMC data of the culture medium samples taken during several fermentations indicated early, large increases in surfactant concentration followed by rapid dissipation. In the fermentation represented by Fig. 1 and 2, there was a close correlation between the behavior of the CMC^{-1} of the whole broth and the amount of corynomycolic acids [R^1—CH(OH)—CHR^2—COOH] present in the whole broth which could be extracted into pentane. Two unidentified neutral lipids, which fluoresced blue under UV irradiation of the TLC plates, were the only other lipids to show concentration maxima at the same time. However, neither was present in more than trace amounts. Thus the production of corynomycolic acids seems to be the cause of the early sharp rise in CMC^{-1}.

Because the corynomycolic acids did not absorb at 258 nm, their production cannot be the cause of the peak in UV absorption observed for the pentane extracts (Fig. 2) at 40 to 45 h. This phenomenon may be due to one or both of the two fluorescent neutral lipids produced at this time. It is interesting that the biosynthesis of corynomycolic acids is thought to involve the condensation of two simple fatty acids to give β-keto acids [R^1—C(O)—CHR^2—COOH], which are reduced to corynomycolic acids (2, 22). These β-keto intermediates are unstable and decarboxylate to ketones if not reduced to product (18). However, a UV spectrum has been reported for a methyl ester derivative, and there is a maximum at about 260 nm (18). The pentane solutions demonstrate the increased concentration of a compound with a UV maximum at 258 nm at the same time that the concentration of the corynomycolic acids is increasing rapidly. Conceivably, one of the unknown neutral lipids was a mixture of the β-keto acids produced as intermediates in corynomycolic acid synthesis.

Although the surfactant concentration (CMC^{-1}) decreases after the initial maximum, there is still an appreciable amount of surfactant present at the end of the fermentation. On several monitored fermentations of *C. lepus*, the values of CMC^{-1} started to increase again late in the fermentation (usually after 70 h). This continuing surface activity is probably due to a combination of lipids, including a very small residue of free corynomycolic acids, the other neutral lipids, and the polar lipids.

The correlation between the CMC^{-1} and TLC data of the pentane-soluble lipids illustrates the importance of changing concentrations of different lipids to the overall surface tension. The first large maximum in CMC^{-1} corresponds to the maximum in corynomycolic acid concentration. The second, small increase in CMC^{-1} corresponds to maxima in the concentrations of the other neutral lipids.

The concentration of polar lipids reached a plateau of maximum concentration between 50 and 60 h, as the corynomycolic acid concentration was becoming negligible. After this time the amount of polar lipids present was about 10 times the amount of neutral lipids. In particular, the lipopeptides accounted for 80 to 90% of the total lipid late in the fermentation. This lipid has been isolated and exhibited surface activity (52 dyn/cm). Thus, the lipopeptide probably accounts for most of the surface activity of the culture medium at the end of the fermentation.

The relative concentrations of the two major biosurfactants produced by *C. lepus* are interesting because the one type, the lipopeptides, is about 50% by weight corynomycolic acids, the other type. The concentration of corynomycolic acids maximized early in the fermentation, and the lipopeptides can be observed in the culture medium at this time. As the concentration of the lipopeptides starts to increase, the free corynomycolic acids disappear from solution. This is consistent with the free corynomycolic acids being removed from the culture medium and incorporated into the lipopeptides.

Some of the lipids isolated have taxonomic significance. The corynomycolic acids produced by *C. lepus* are typical of the β-hydroxy α-branched fatty acids found in *Corynebacterium* and related bacteria (2, 14). The phospholipid composition, in particular the complete absence of phosphatidyl ethanolamine, is characteristic of the low-guanine-cytosine-content (DNA) group of *Corynebacterium* (4, 16, 19).

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